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## Deflavination of flavo-oxidases by nucleophilic reagents

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### Abstract

Using spectroscopic techniques we studied the effect of the nucleophilic reagents cyanide, cyanate and thiocyanate on three flavo-oxidases namely alcohol oxidase (AO), glucose oxidase (GOX) and D-amino acid oxidase (DAOX). All three ions, added at concentrations in the mM range, caused release of the flavin adenine dinucleotide (FAD) co-factors from the enzyme molecules. In the case of AO this was accompanied by significant conformational perturbations, which was not observed for GOX and DAOX. As suggested from fluorescence, absorption and circular dichroism spectral changes at least one phenolic hydroxyl group became ionized upon FAD release from AO and a new class of Trp residues, fluorescent only in apo-AO protein, was demasked. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Flavo-oxidase; Fluorescence; Circular dichroism; Nucleophilic reagent; Co-factor

### 1. Introduction

Flavo-enzymes are widespread in nature and involved in a diversity of chemical reactions [1]. An important function of flavo-enzymes is the catalysis of redox reactions. Alcohol oxidase (AO), glucose oxidase (GOX) and D-amino acid oxidase (DAOX) are enzymes that belong to this group. These en-

zymes bind flavin adenine dinucleotide (FAD) non-covalently and contain the well-characterized Rossmann fold [2] with the  $\beta\alpha\beta$  motif involved in binding of the ADP moiety of FAD [3–5]. Flavo-oxidases that covalently bind FAD also exist. These enzymes, however, contain another conserved FAD-binding domain [6].

Previous studies on AO revealed that the FAD co-factor is tightly bound and only dissociates in vitro upon complete denaturation of the protein after incubation in a solution containing 6 M urea and 3.5 M KBr [7]. In GOX or DAOX the FAD molecule is much weaker bound and readily dissociates under much milder conditions [8,9]. Moreover, the enzyme activity of both GOX and DAOX FAD-depleted states can be fully recovered upon addition of FAD

Abbreviations: AO, alcohol oxidase; GOX, glucose oxidase; DAOX, D-amino acid oxidase; FAD, flavin adenine dinucleotide; CD, circular dichroism; CN<sup>−</sup>, cyanide; CNO<sup>−</sup>, cyanate; SCN<sup>−</sup>, thiocyanate

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whereas all attempts to re-activate apo-AO failed so far [9–12]. The observed differences in FAD-binding clearly show variations in the accessibility, size and geometry of the FAD-binding pockets, most probably related to the specific types of chemical reactions catalyzed by these flavo-enzymes.

Previously, we observed that treatment of intact *Hansenula polymorpha* cells with cyanide (in mM concentrations) resulted in the dissociation of the FAD co-factor of the AO enzyme but not in dissociation of the octameric enzyme into monomers [13]. The mechanism behind this phenomenon, however, remained obscure. Spectroscopic characterization of both octameric and monomeric *H. polymorpha* and *Pichia pastoris* AO showed significant conformational perturbations of the molecules upon FAD release thus demonstrating the importance of this co-factor for the structural integrity of the enzymatically active conformations of the two enzymes [14].

In this paper we present a detailed spectroscopic analysis of the dissociation of FAD from AO mediated by KCN and other nucleophilic reagents in vitro. In addition we show that these reagents also cause dissociation of the FAD co-factor from two other structurally related flavo-oxidases, namely GOX and DAOX.

## 2. Materials and methods

### 2.1. Proteins

Native *P. pastoris* AO protein was obtained from Provesta Corporation (Bartlesville, OK, USA). AO from *H. polymorpha* was purified as described previously [15]. *Aspergillus niger* GOX and porcine kidney DAOX were obtained from Sigma (Saint Louis, MO, USA). All protein solutions were in 50 mM potassium phosphate buffer, pH 7.5. Dissociation of native AO octamers into monomers was achieved in 60% glycerol [14,16]. *H. polymorpha* and *P. pastoris* AO, GOX and DAOX were depleted of FAD upon incubation with 6 mM of either KCN, NaSCN or KCNO for 60–80 min at 10°C followed by dialysis for 24 h at 10°C against phosphate buffer, pH 7.5. Solutions of monomeric AO were dialyzed for 72 h against the same phosphate buffer, supplemented with 60% glycerol.

### 2.2. Spectroscopic measurements

Absorption spectra were recorded using a Beckman spectrophotometer and corrected for light scattering when necessary. Steady-state fluorescence was measured using a Shimadzu model RF 5000 spectrofluorometer equipped with a temperature-controlled cuvette holder. Excitation was at 275 nm for Tyr and Trp residues and, selectively, at 295 nm for Trp chromophores. Fluorescence of the FAD co-factor was excited at 450 nm. The protein absorbance at the excitation wavelengths was always lower than 0.05 in order to minimize inner filter and self-absorption effects. Excitation spectra were recorded at 375 nm emission wavelength. The emission spectra obtained in the presence of cyanate ( $\text{CNO}^-$ ) were corrected for inner filter effects due to the absorption of these ions at the excitation wavelengths ( $\epsilon_{\text{M}, 275} = 21$  and  $\epsilon_{\text{M}, 295} = 3$ ). Circular dichroism (CD) spectra in the near-UV region (250–325 nm) of the by KCN depleted octameric and monomeric states of AO were recorded as described in [14].

### 2.3. Miscellaneous

AO [17] and DAOX [18] were assayed as described previously. Rebinding of FAD to apo-DAOX was achieved by addition of FAD (2  $\mu\text{M}$  final concentration) to the assay mixture.

## 3. Results

### 3.1. KCN, KCNO or NaSCN cause dissociation of FAD from AO

We previously showed that incubation of intact *H. polymorpha* cells with 6 mM KCN resulted in the inactivation of AO due to the irreversible dissociation of the co-factor FAD [13]. In order to study whether this was an indirect effect due to the change in energy state of the cells (ATP depletion due to KCN) or a direct effect of KCN on AO protein, we tested whether AO inactivation and FAD dissociation also occurred after treatment of purified AO with KCN in vitro.

To this purpose we incubated purified AO enzyme with 6 mM KCN, followed by dialysis against fresh

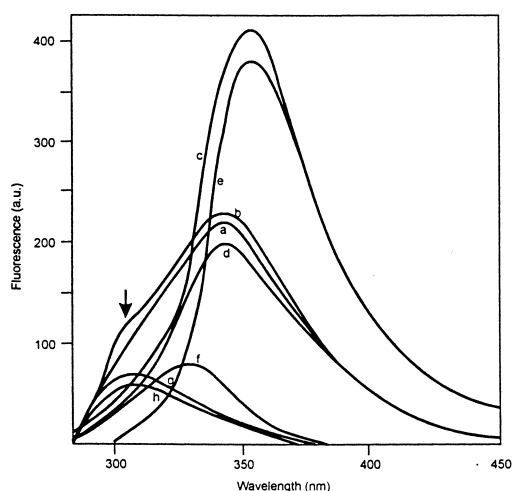


Fig. 1. Fluorescence emission spectra of *H. polymorpha* AO excited at 275 nm (a, b, c) and at 295 nm (d, e). Spectra a and d represent the native, untreated enzyme, spectrum b was obtained in the presence of KCN and spectra c and e after dialysis with fresh buffer lacking KCN. The spectra h (untreated protein), g (protein in the presence of KCN) and f (after dialysis of KCN-treated protein sample) are obtained by subtraction of the spectra excited at 295 nm from those excited at 275 nm.

buffer without KCN. Enzyme activity measurements revealed that after incubation of AO in the presence of KCN for 1 h, the enzyme activity of AO was significantly reduced. Upon subsequent dialysis of the KCN-treated protein against fresh buffer lacking KCN, the enzyme remained inactivated.

In order to analyze the effect of KCN on AO protein, we recorded fluorescence emission (Fig. 1) and excitation spectra (Fig. 2) of untreated and cyanide-treated AO protein samples. In addition samples were analyzed that were incubated with cyanide and subsequently dialyzed against fresh buffer. The emission spectra of the protein obtained either at 275 or 295 nm excitation in the absence or presence of KCN were completely dominated by Trp fluorescence (Fig. 1, spectra a, b and d). Upon addition of KCN, however, a shoulder appeared at 305 nm in the emission spectrum excited at 275 nm (Fig. 1, b, arrow) indicating dequenching of Tyr chromophores whereas the Trp emission remained unchanged (see Fig. 1, represented by spectrum 'd' in both the untreated and cyanide-treated samples). The contribution of Tyr residues was obtained by subtracting the Trp emission spectrum excited at 295 nm from the emission spectrum excited at 275 nm after nor-

malizing the two spectra above 380 nm where the Tyr emission is negligible. As shown in Fig. 1, spectra h and g, addition of KCN caused a slight increase of the Tyr contribution to the overall protein emission.

The excitation spectrum obtained at a fixed emission wavelength of 375 nm after the addition of cyanide was essentially similar to that of the native enzyme (Fig. 2, curves a and b, respectively). The cyanide ions did also not affect the specific FAD fluorescence in *H. polymorpha* AO as seen from spectra a and b in Fig. 3, which indicate that the FAD co-factor remained bound to the protein in the presence of the KCN. However, upon dialysis of the cyanide-treated sample the FAD co-factor was removed and the apo-state of AO obtained, as indicated by the disappearance of the flavin emission (Fig. 3, c) and absorbance (data not shown) bands.

FAD release was associated with a drastic increase of the fluorescence emission intensity and an approximately 14 nm red-shift of the emission maximum position (Fig. 1, compare c with a and b, and e with d, respectively). It also caused significant changes in the intensity and shape of the fluorescence excitation spectrum evident from 4 and 20 nm shifts of the excitation maximum and minimum, respectively, to the longer wavelengths (red-shift, Fig. 2, compare c with a and b) as well as the appearance

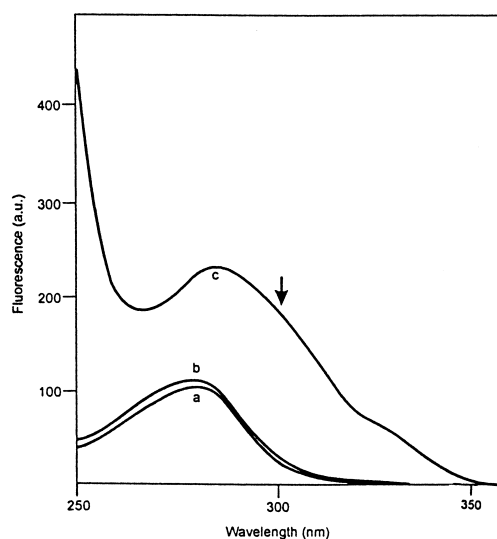


Fig. 2. Fluorescence excitation spectra of *H. polymorpha* AO. Spectrum a, untreated sample; spectrum b, sample treated with KCN; spectrum c, KCN-treated and dialyzed sample. The emission wavelength for all spectra was fixed at 375 nm.

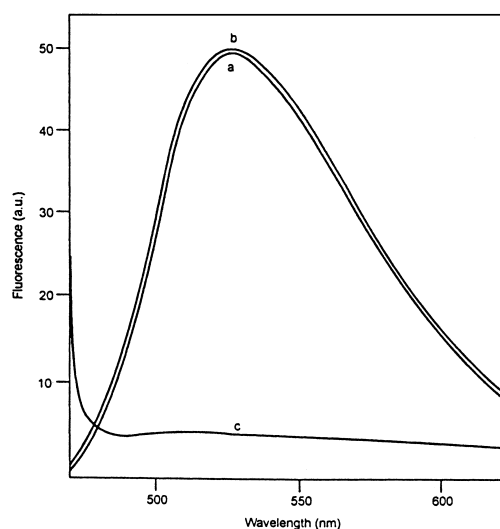


Fig. 3. FAD fluorescence emission spectra of *H. polymorpha* AO, excited at 450 nm. Spectrum a is of the native enzyme, b is obtained in the presence of KCN and c after dialysis of the treated sample.

of a shoulder at 295 nm (Fig. 2, spectrum c, arrow). Whereas the ratio between the maximal fluorescence intensities at 275 and 295 nm excitation ( $F_{\max,275}/F_{\max,295}$ ) was approximately two in the spectra of the enzyme registered in the absence or presence of cyanide ions (Fig. 2, a and b) this value amounted one when the FAD-depleted AO was analyzed (Fig. 2, spectrum c). These alterations indicate a possible contribution of ionized Tyr residues to the protein emission spectra after FAD release. In contrast to Trp or Tyr, the tyrosinate state should emit stronger at 295 than at 275 nm excitation, as its absorption maximum is at 295 nm and thus, approximately 15–20 nm shifted to red in comparison with that of either Trp or Tyr residues.

Differential computation of the emission spectra of FAD-depleted AO proteins excited at 275 (where both Tyr and Trp chromophores absorb) and 295 nm (where predominantly Trp absorbs) resulted in spectra with emission maximum at approximately 325–330 nm (Fig. 1, curve f), a position characteristic of buried Trp chromophores. This observation suggests dequenching of the fluorescence of some Trp residues in the FAD-depleted state of the protein. No tyrosine emission could be registered in this state. Essentially similar results were obtained when AO protein from the related yeast *P. pastoris* was used (data not shown).

In experiments using *H. polymorpha* or *P. pastoris* AO and 6 mM of either NaSCN or KCNO instead of KCN, essentially similar results were obtained. Hence, not only cyanide, but also other nucleophilic ions like thiocyanate and cyanate cause destabilization of FAD-binding and favor dissociation of FAD during subsequent dialysis of the treated protein sample.

We previously studied the conformational properties and stability of the octameric and monomeric

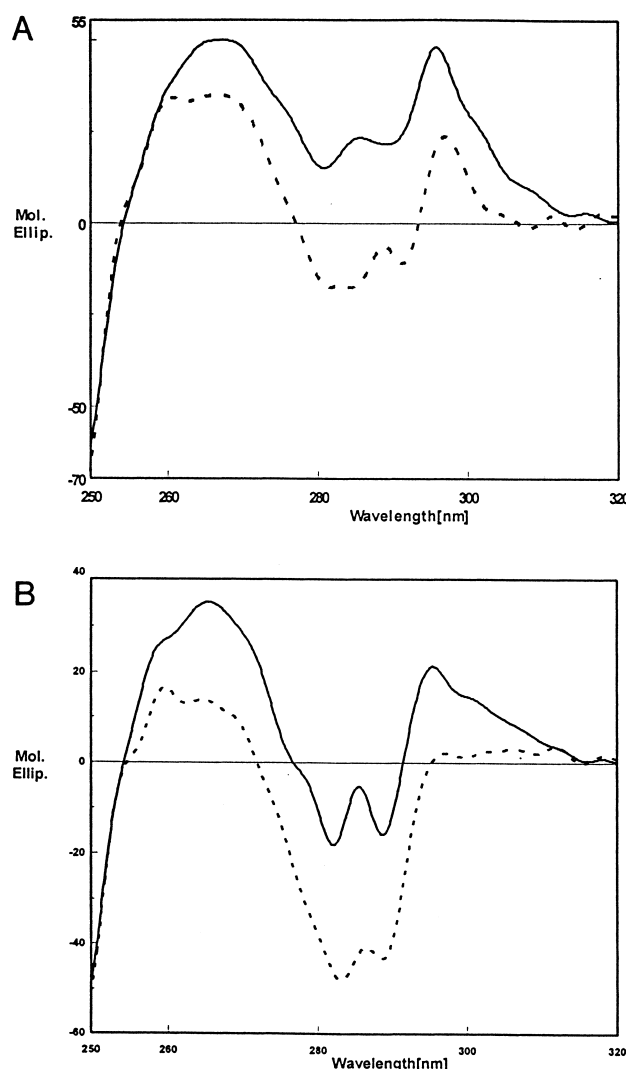


Fig. 4. CD spectra in the near-UV region (250–325 nm) of FAD depleted (apo-state) *H. polymorpha* (A) and *P. pastoris* AO (B). The solid lines represent the spectra of the apo-octamers and the dashed lines of the apo-monomers. The octamers are measured in buffer and the monomers in buffer supplemented with 60% glycerol.

states of AO and found dramatic differences between both conformations [14]. Therefore, we checked whether the effects of the three nucleophilic reagents (KCN, NaSCN and KCNO) on AO protein were dependent on the oligomeric state or affected the FAD-binding in monomers as well. For this purpose, *P. pastoris* AO was first dissociated into subunits by treatment with 60% glycerol [14,16], subsequently  $\text{CN}^-$ ,  $\text{SCN}^-$  or  $\text{CNO}^-$  ions were added and finally – upon incubation for 60 min – dialyzed against glycerol-containing buffer in order to keep the protein in the monomeric conformation. The nucleophilic reagents caused dissociation of FAD and similar spectroscopic changes as those observed with the octamers (data not shown). Only slight differences between the octameric and monomeric states with regard to fluorescence intensities of the aromatic residues were registered.

The CD spectra of the FAD-depleted octamers and monomers of *H. polymorpha* and *P. pastoris* AO (Fig. 4) in the near-UV or aromatic range (250–325 nm) indicative for the three-dimensional (3D) structure of the molecules consist of two positive maxima at approximately 295 and 260–270 nm and two minima at 289 and 283 nm. AO proteins from both yeast species do not contain disulfide bridges, hence the CD bands are attributed to the aromatic residues mainly. The two negative bands at 289 and 283 nm arise most probably from the Trp  $^1\text{L}_b$  electronic transition characterized by absorption up to 290 nm and by a fine CD structure. They may reflect the contribution of Tyr residues, which absorb in this part of the spectrum as well. The bands at 295 and 260–270 nm could originate from the  $^1\text{L}_a$  transition of Trp which is avoided of fine structure and usually overlaps both the long and short wavelength ends of the  $^1\text{L}_b$  band with higher intensity concentrated at 265 nm. Alternatively, the band at 295 nm could be related to a tyrosinate state whose absorption maximum position supports this attribution. Differences in the CD spectra of the FAD-depleted octamers and monomers were expressed mainly in changes in the intensity of all CD bands and disappearance of the band at 295 nm in the spectrum of *P. pastoris* apo-monomers.

### 3.2. Effect of KCN, NaSCN and KCNO on FAD-binding in GOX and DAOX

Next we tested whether the three nucleophilic reagents affect FAD-binding in other flavo-oxidases like GOX from *A. niger* and porcine DAOX. Both proteins were characterized by remarkably similar fluorescence spectra, completely dominated by the Trp chromophores with maximum at approximately 340 nm (data not shown). Incubation of either of the two enzymes with 6 mM KCN, NaSCN or KCNO and subsequent dialysis resulted in the release of their FAD co-factors, akin to the observations made for AO. In contrast to AO, however, both native and FAD-depleted GOX and DAOX enzymes displayed almost identical fluorescence emission and excitation spectra indicative of similar conformations of their native and apo-states. Remarkably, no tyrosinate emission resulting from the ionization of tyrosine residues upon FAD release was observed. The enzyme activity of apo-DAOX obtained by KCN treatment and subsequent dialysis could be fully recovered by the addition of excess FAD (data not shown) indicating that FAD easily reassociated to apo-DAOX, which was obtained by this procedure.

## 4. Discussion

The 3D structures of GOX and DAOX show topological similarities with regard to the FAD-binding pocket [3,5,19]. Also AO, which displays sequence homology with GOX (approximately 25% identity), may have a similar structure [14]. According to the 3D structure of GOX the FAD molecule is embedded in a narrow channel of the protein matrix and forms a number of hydrogen bonds and salt bridges with a number of protein side chains [19]. The network of non-covalent chemical bonds increases the polarization of the functional groups involved in the FAD-binding and makes them more susceptible to the attack of nucleophilic ions like cyanide, cyanate and thiocyanate. These reagents are even capable of chemical reactions with amino, sulfhydryl, imidazole or phenolic hydroxyl groups or

with phosphate anions of the FAD co-factor [20]. In GOX several residues containing such side chains were found to interact with FAD and are therefore likely candidates to react with the nucleophilic reagents [19]. Apparently, the non-covalent bonds involved in the FAD-binding are destabilized during incubation of the proteins with KCN, NaSCN or KCNO resulting in inactivation of the enzymes, but not in dissociation of the co-factor from the proteins. The latter occurred, however, when the treated protein samples were dialyzed against fresh buffer in order to remove the reagents added. In the present study we show that each of the three nucleophilic reagents tested (cyanide, cyanate or thiocyanate) can release the FAD molecule from either AO, GOX or DAOX at concentrations in the mM range.

Addition of the nucleophilic reagents did not result in major alterations in the fluorescence emission or excitation spectra of AO. In contrast, upon subsequent dialysis dramatic changes in the spectroscopic properties of the enzyme were observed. These findings indicate that the changes were not caused by the reagents, but instead were due to significant conformational perturbations of the AO molecules. These perturbations were accompanied by the dissociation of the FAD co-factor, resulting in the formation of the apo-form of the enzyme.

Judged from the changes in the emission, absorption and CD spectra of *H. polymorpha* and *P. pastoris* apo-AO at least one phenolic hydroxyl group became ionized after FAD removal. Normally, the tyrosinate emission quantum yield is extremely low. However, in the proteins a non-radiative Trp to tyrosinate energy transfer is possible due to a significant overlap of the Trp emission and tyrosinate absorption bands and this process could significantly increase the tyrosinate emission intensity.

The neutral pH at which tyrosinate is registered is relatively far from the ionization pH range typical of the Tyr side chain. However, there are several examples of abnormal pK values near pH 7 for Tyr residues hydrogen-bonded to protein functional groups [21–23]. In particular, the 3D model of vanillyl-AO shows that a tyrosine (Tyr 187) is involved in a hydrogen bond with the flavin ring and that two other Tyr residues (Tyr 108 and 503) are part of the catalytic site of the enzyme [6]. Similar topology and participation of two tyrosine residues (Tyr 223 and

228) in the substrate-binding site of DAOX was also found in the very high-resolution structure of the enzyme [24]. The analysis of the FAD steady-state fluorescence, fluorescence and anisotropy decays of *H. polymorpha* and *P. pastoris* AO [14] also indicated interactions of their flavin moieties with aromatic side chains from the FAD-binding pocket which led to a strong quenching of the flavin fluorescence. Accordingly, at least two Tyr residues could be identified in the FAD-binding site which are probably involved in excited-state interactions with the flavin molecule. Similar charge-transfer complexes between FAD and Tyr residues leading to a very short fluorescence lifetime pattern have been shown to exist in other flavoproteins as well [25,26]. Quite interestingly, FAD could be even covalently bound to a tyrosine residue as found in the flavocytochrome subunit of *p*-cresol methylhydroxylase [27].

Similar to Tyr, Trp residues could also be involved in charge-transfer reactions with the flavin ring resulting in quenching of both FAD and Trp fluorescence. The present study confirmed this and showed that release of FAD from both *H. polymorpha* and *P. pastoris* AO enzymes demasks a new class of Trp residues characterized by a blue-shifted emission with a maximum at 325–330 nm and a negligible absorbance at 295 nm. It has been found that Trp moieties interacting with negatively charged functional groups localized in their neighborhood (e.g. carboxylic groups of glutamic and aspartic acids) display very low, ‘anomalous’ absorption in the 290–300 nm region [28]. This effect was assigned to the electrostatic repulsion of electrons of the indole nitrogen atom by the carboxylic groups. Therefore, the Trp emission observed at the blue end of the spectrum suggests that some acidic residues (Asp or Glu) participate in electronic interactions with Trp residues.

In contrast to AO, both native and FAD-depleted GOX and DAOX displayed largely similar conformations, which is probably a prerequisite for the proper flavin attachment requiring a favorable approach between the flavin ring and the corresponding reactive residues of the polypeptide chain. An alternative explanation that FAD can be rebound to GOX and DAOX, but not to FAD-depleted AO, may be that in AO, but not in GOX and DAOX, KCN treatment resulted in the formation of tyrosinate, which may hamper FAD-rebinding.

In conclusion, the present study shows that in spite of the same folding topology of the three flavin-dependent oxidases studied (AO, GOX and DAOX), subtle differences in the reactivity, architecture and size of the active site cavity exist. The diversity is probably of utmost importance for the proper control of their substrate specificities by providing a 'size exclusion mechanism', a rule addressing a number of flavin-dependent oxidases [6]. Nevertheless, mM concentrations of each of the three nucleophilic reagents cyanide, cyanate and thiocyanate can release under mild conditions the FAD co-factors of AO, GOX and DAOX.

## References

- [1] D.R. Cavener, *J. Mol. Struct.* 223 (1992) 811–814.
- [2] R.K. Wierenga, J. Drenth, G.E. Schulz, *J. Mol. Biol.* 167 (1983) 725–739.
- [3] A. Vrielink, L.F. Lloyd, D.M. Blow, *J. Mol. Biol.* 219 (1991) 533–554.
- [4] J. Li, A. Vrielink, P. Brick, D.M. Blow, *Biochemistry* 32 (1993) 11507–11515.
- [5] A. Mattevi, M.A. Vanoni, F. Todone, M. Rizzi, A. Teplya-kov, A. Coda, M. Bolognesi, B. Curti, *Proc. Natl. Acad. Sci. USA* 93 (1996) 7496–7501.
- [6] M.W. Fraaije, W.J.H. van Berkel, J.A.E. Benen, J. Visser, A. Mattevi, *Trends Biochem. Sci.* 23 (1998) 206–207.
- [7] I.J. van der Klei, W. Harder, M. Veenhuis, *Yeast* 7 (1991) 195–209.
- [8] V. Massey, B. Curti, *J. Biol. Chem.* 241 (1966) 3417–3423.
- [9] L. Pollegioni, M.S. Pilone, *Arch. Biochem. Biophys.* 332 (1996) 58–62.
- [10] P. Casalin, L. Pollegioni, B. Curti, M.S. Pilone, *Eur. J. Biochem.* 197 (1991) 513–517.
- [11] L. Pollegioni, F. Ceciliani, B. Curti, S. Ronchi, M.S. Pilone, *Biochem. J.* 310 (1995) 577–583.
- [12] A. Stocker, H.-J. Hecht, A.F. Buckmann, *Eur. J. Biochem.* 238 (1996) 519–528.
- [13] I.J. van der Klei, M. Veenhuis, K. Nicolay, W. Harder, *Arch. Microbiol.* 151 (1989) 26–33.
- [14] R. Boteva, A.J.W.G. Visser, B. Filippi, G. Vriend, M. Veenhuis, I.J. van der Klei, *Biochemistry* 38 (1999) 5034–5044.
- [15] I.J. van der Klei, L.V. Bystrykh, W. Harder, *Methods Enzymol.* 188 (1990) 420–427.
- [16] M. Evers, W. Harder, M. Veenhuis, *FEBS Lett.* 368 (1995) 293–296.
- [17] C. Verduyn, J.P. van Dijken, W.A. Scheffers, *J. Microbiol. Methods* 2 (1984) 15–25.
- [18] G.J. Sulter, I.J. van der Klei, W. Harder, M. Veenhuis, *Yeast* 6 (1990) 501–509.
- [19] H.J. Hecht, H.M. Kalisz, J. Hendle, R.D. Schmid, D. Schomburg, *J. Mol. Biol.* 229 (1993) 153–172.
- [20] G.R. Stark, *Methods Enzymol.* 11 (1967) 590–595.
- [21] E. Bicknel-Brown, B.T. Lym, T. Kumura, *Biochem. Biophys. Res. Commun.* 101 (1981) 298–305.
- [22] J. Jordano, J.L. Barbero, F. Montero, L. Franco, *J. Biol. Chem.* 258 (1983) 315–320.
- [23] A.G. Szabo, K.R. Lynn, D.T. Krajcarsky, D.M. Rayner, *FEBS Lett.* 94 (1978) 249–252.
- [24] S. Umhau, L. Pollegioni, G. Molla, K. Diederichs, W. Welte, M.S. Pilone, S. Ghisla, *Proc. Natl. Acad. Sci. USA* 97 (2000) 12463–12468.
- [25] P.I.H. Bastiaens, A. van Hoek, W.F. Wolkers, J.C. Brochon, A.J.W.G. Visser, *Biochemistry* 31 (1992) 7050–7060.
- [26] P.A.W. van den Berg, A. van Hoek, C.D. Walentas, R.N. Perham, A.J.W.G. Visser, *Biophys. J.* 74 (1998) 2046–2058.
- [27] J. Kim, J.H. Fuller, V.L. Kuusk, Z. Cunane, W. Chen, F.S. Mathews, W.S. McIntire, *J. Biol. Chem.* 270 (1995) 31202–31209.
- [28] P. Elödi, S. Lakatos, *Eur. J. Biochem.* 36 (1973) 45–52.